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Nitrogen Fixation (*nif*) Genes and Large Plasmids of *Rhizobium japonicum*

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The location of structural nitrogen-fixation genes was determined for the slow- and fast-growing types of *Rhizobium japonicum*. Slow-growing *R. japonicum* strains do not harbor structural *nif* genes, homologous to *nifD* and *nifH*, on large plasmids (100 to 200 megadaltons). In contrast, all fast-growing *R. japonicum* strains, except PRC194, contain structural *nif* genes on large plasmids.

The symbiotic bacterium *Rhizobium japonicum* inhabits and fixes atmospheric nitrogen in the root nodules of soybeans. *Rhizobium* species include fast- and slow-growing types, of which *R. japonicum* is a slow-growing species with a doubling time of 6 to 13 h. Recently, however, fast-growing *R. japonicum* strains (doubling times of 2 to 4 h) from the People's Republic of China have been examined. These strains physiologically resemble fast-growing *Rhizobium* species and yet still nodulate soybean plants (10). Keyser et al. (10) found that fast-growing *R. japonicum* form symbiotic relationships with the soybean cultivar Peking but generally are ineffective with common North American soybean cultivars.

A common feature of both slow- and fast-growing *R. japonicum*, as well as other *Rhizobium* species, is the presence of large plasmids (between 90 and 250 megadalton [mdal]) (2, 4, 7, 12, 14, 17). The slow-growing *R. japonicum* strains examined in this study usually contained one large plasmid ranging between 118 and 196 mdal, whereas the fast-growing strains contained one to three plasmids ranging from 54 to 197 mdal. Therefore, large plasmids are a consistent feature in both slow- and fast-growing *R. japonicum*. In addition, almost all fast-growing *Rhizobium* strains examined have been found to possess very large plasmids. These plasmids have been referred to as megaplasmids. *nif* and *nod* genes have been found on megaplasmids in some strains (3); however, megaplasmids have never been found in slow-growing *Rhizobium* strains. Because of the location of nodulation and nitrogen fixation genes on large plasmids in *Rhizobium* species (7, 8, 11, 13, 14, 18, 19), it is of interest to determine the genetic content of plasmids in these strains to further our understanding of *R. japonicum*-soybean symbiosis.

Table 1 contains the molecular weights of large plasmids separated from slow- and fast-growing *R. japonicum* species as well as molecular weight standards. Considerable difficulty has been reported in the isolation of intact covalently closed circular DNA from slow-growing *R. japonicum* (3). This is partly due to the high degree of resistance to lysis and the large size of the plasmids in these strains. However, the isolation procedures of Casse et al. (2) and Hirsch et al. (7) have worked well in the isolation of slow- and fast-growing *Rhizobium* species, with only slight modification. The plasmid yields of slow-growing *R. japonicum* were enhanced when cultures were prewashed in 3% NaCl before isolation. Isolated plasmids were suspended in 10 mM Tris-hydrochloride (pH 8.0) and applied to a 0.7% vertical gel. Electrophoresis was conducted in TBE (pH 8.3; 10.8 g of Tris base, 5.5 g of boric acid, and 0.93 g of EDTA per liter) at 100 V for 5 h at 6 to 8°C. The gels were then stained in ethidium bromide (1 μg/ml) and photographed with a Kodak MP4 camera apparatus and Poloroid 665 film. Gels suitable for Southern transfer blotting were irradiated with short-wave UV light for 15 min to produce nicks in the large plasmids such that a transfer to nitrocellulose would be maximized. The procedure of Southern (20), modified by Haugland and Verma (6), was used in the transfer, hybridization, and autoradiography of plasmids listed in Table 1. The plasmid pRmR2 (a gift of G. Ruvkun and F. Ausubel, Harvard University, Cambridge, Mass.), containing the structural *nif* genes was isolated and purified in a CsCl-ethidium bromide gradient according to the method of Hirsch et al. (7). A 1-μg sample of plasmid pRmR2 was nick translated according to the procedure of Rigby et al. (15) and was allowed to hybridize at 65°C for 48 h with the nitrocellulose sheets (Millipore HAHY) containing *Rhizobium* and *Agrobacterium* plasmids.

TABLE 1. Properties of large plasmids

Strain of origin	Plasmid(s)	Mol wt	Hybridization to pRmR2
R. japonicum[a] (slow-growing type)			
61A76	pRja61A76	178 ± 4	−
AA102	pRjaAA102	138 ± 6	−
3Ilb31	pRja3Ilb31	160 ± 11	−
3Ilb71a	pRja3Ilb71a	164 ± 2	−
3Ilb74	pRja3Ilb74	195 ± 6	−
3Ilb94	pRja3Ilb94a	58 ± 9	−
	pRja3Ilb94b	118 ± 6	−
3Ilb110	pRja3Ilb110	184 ± 7	−
3Ilb143	pRja3Ilb143	159 ± 6	−
R. japonicum (fast-growing type)			
PRC194	pRjaPRC194a	76 ± 13	−
	pRjaPRC194b	145 ± 5	−
PRC201	pRjaPRC201a	117 ± 4	−
	pRjaPRC201b	192 ± 25	+
PRC205	pRjaPRC205a	57 ± 15	−
	pRjaPRC205b	112 ± 3	+
	pRjaPRC205c	192 ± 25	−
PRC206	pRjaPRC206a	54 ± 12	−
	pRjaPRC206b	60 ± 8	−
	pRjaPRC206c	197 ± 27	+
PRC440	pRjaPRC440a	69	−
	pRjaPRC440b	195	+
Ob3	pRjaOb3	186	+
R. meliloti[b]			
102F28	pRme102F28a	73 ± 4	−
	pRme102F28b(c)	118 ± 3	−
102F51	pRme102F51	93 ± 3	+
R. phaseoli[c]			
DB1	pRphDB1a	166	−
	pRphDB1b	248	+
A. tumifaciens[b]			
A227	pTi	119 ± 3	−
	Cryptic	300	−

[a] See reference 17 for molecular weight determinations.
[b] See reference 2.
[c] From D. Berryhill, North Dakota State University, Fargo.

Colony hybridizations were performed according to the method of Grunstein and Hogness (5) and hybridized under the same conditions as the separated plasmids.

The structural *nifKDH* genes and part of the *nifE* genes have been cloned from *Klebsiella pneumoniae* into plasmid pSA30 (1). The *nifD* and *nifH* genes, which code for a subunit of nitrogenase and nitrogenase reductase, respectively, exhibit a high degree of homology with all nitrogen-fixing organisms examined (18). Ruvkun and Ausubel (19) used pSA30 as a probe to isolate the structural *nif* genes from *R. meliloti*. A recombinant plasmid, pRmR2, was obtained, which included a 3.9-kilobase *Eco*R1 fragment homologous to the *nifD* and *nifH* genes in *K. pneumoniae*. Using purified nick translated (15) pRmR2 plasmid (specific activity, 6×10^7 cpm per μg of DNA) we examined colony hybridization (5) to total DNA as well as Southern hybridization (20) of separated plasmid DNA for strains listed in Table 1. No hybridization was obtained by using ^{32}P-labeled plasmid pRmR2 as the probe to endogenous plasmids of slow-growing strains. Lanes 9 through 10′ in Fig. 1 are representative samples of negative results. No hybridization is seen in lanes 9′ and 10′ to either strain 61A76 or AA102. Repeated hybridization attempts yielded identical results. However, colony hybridization of pRmR2 to total DNA of the

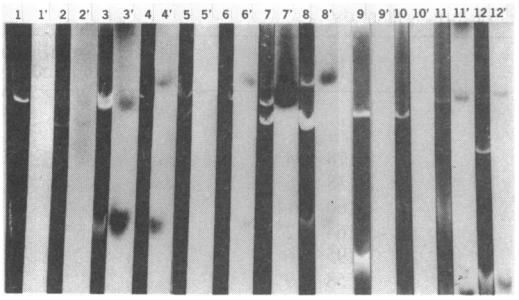

FIG. 1. Hybridization of ^{32}P-labeled *R. meliloti nif* DNA to plasmids listed in Table 1. Numbers 1 through 12 (unprimed) represent eithidium bromide strained plasmids separated by 0.7% agarose gel electrophoresis; primed numbers are autoradiograph results of hybridization. See Table 1 for plasmid content and molecular weights. Lane 1, *A. tumifaciens* A277; lane 2, *R. meliloti* 102F28; lane 3, *R. meliloti* 102F51; lane 4, *R. phaseoli* DB1; lane 5, *R. japonicum* PRC194; lane 6, *R. japonicum* PRC201; lane 7, *R. japonicum* PRC205; lane 8, *R. japonicum* PRC206; lane 9, *R. japonicum* 61A76; lane 10, *R. japonicum* AA102; lane 11, *R. japonicum* OB3; and lane 12, *R. japonicum* PRC440.

slow-growing strains yielded positive hybridization results. *Agrobacterium tumefaciens* (a nonnitrogen fixing member of the *Rhizobiaceae*) was used as a negative control. Therefore, hybridization occurs with total DNA of these strains, not with the separated plasmids. These results agree with the findings of Haugland and Verma (6). They concluded that *R. japonicum* strains 61A76 (lanes 9 and 9') and 3I1b110 structural *nif* genes were not present on large plasmids by using plasmid pSA30 as a probe.

Of the fast-growing *R. japonicum* strains listed in Table 1, most appear to contain *nif* genes located on large plasmids. Strains PRC201 (lanes 6 and 6'), PRC206 (lanes 8 and 8'), PRC440 (lanes 12 and 12'), and Ob3 (lanes 11 and 11') all contain structural *nif* genes on their largest plasmid. Strain PRC205 contains structural *nif* genes on an intermediate 112-mdal plasmid (lanes 7 and 7' of Fig. 1). The only fast-growing *R. japonicum* strain not containing *nif* structural genes on a plasmid is strain PRC194 (lanes 5 and 5'). Colony hybridization results indicated that the *nif* genes are present in strain PRC194. Control hybridization with nick translated plasmid pACYC184, the plasmid vector without the *nif* genes as in plasmid pRmR2, showed homology only to blotted pRmR2. Note in lanes 1 and 1' that no hybridization occurred with the plasmids of *A. tumefaciens* strain A277. Therefore, these results indicate specific hybridization between structural *nif* genes in pRmR2 and the *Rhizobium* strains examined.

We also examined hybridization of plasmid pRmR2 to the plasmids in *R. phaseoli* strain DB1. The large 248-mdal plasmid (lanes 4 and 4') was found to possess homologous *nif* genes. In *R. meliloti* strain 102F51 (lanes 3 and 3'), the 93-mdal plasmid also was found homologous to pRmR2. Jouanin et al. (9), however, did not find hybridization with plasmid pSA30 to the 93-mdal endogenous plasmid of strain 102F51. It is possible that plasmid pRmR2 may contain non-*nif* sequences homologous to *R. meliloti* strain 102F51; however, this seems unlikely since no hybridization occurred with *R. meliloti* strain 102F28 (lanes 2 and 2').

Rosenberg et al. (16) have reported the presence of megaplasmids in *R. meliloti* with molecular masses greater than 450 megadaltons. Plasmid pRmR2 hybridized to a megaplasmid of one *R. meliloti* strain, indicating the presence of structural *nif* genes; thus, although structural *nif* genes have not been found on the large plasmids of slow-growing *R. japonicum*, it is conceivable these genes may be located on megaplasmids. Preliminary evidence suggests the presence of megaplasmids in slow-growing *R. japonicum* (unpublished data) and the presence of *nif* genes on these large plasmids will be investigated in future studies.

In conclusion, it is interesting that *nif* genes appear mostly on the plasmids of fast-growing *R. japonicum* strains and, very likely, only on the chromosome or megaplasmids of slow-growing strains. Ruvkun and Ausubel (18) speculate that plasmid-borne, nitrogen-fixation-related traits may be the result of recent radiation, perhaps by conjugative plasmids to other bacterial species. The acquirement of nitrogen-fixation genes would allow an organism new niches, including symbiotic interaction. Thus, it is possible that the fast-growing *R. japonicum* strains isolated from the People's Republic of China are relatively recent additions to the family of nitrogen-fixing organisms. The isolation and cloning of the plasmids in this study are in progress, and the characterization of *nif* and other genes present should produce valuable information about these symbionts.

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